Feeding different forms of methionine and rumen-protected methionine alters the incorporation of ^{15}N into microbial protein in batch culture

Thesis

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Abstract

Creating a balanced amino acid (AA) profile for rumen microbes increases efficiency of feed conversion into microbial protein , which decreases manure N. Methionine (MET) is a limiting AA in lactating dairy cows. A steady supply of MET reduces the need for rumen bacteria to synthesize AA from carbon skeletons.

A previous study conducted in the OSU dairy lab evaluated the supplementation of 2-hydroxy-4-(methylthio) butanoic acid (HMB) isopropyl ester (HMBi; 0.11%), MET (0.097%), HMBi + MET (0.055% + 0.048%) and control (CON) to continuous culture (CC) fermenters. Extracellular MET pools were higher for HMBi treatments than MET, but microbial protein synthesis (MPS) did not differ.

We hypothesized MET accumulation may be due to differences in bacterial utilization of MET stereoisomers, because isopropanol (ISO; hydrolyzed from HMBi) may affect membrane fluidity and HMBi is more slowly converted to MET. This expanded treatments (TRT) to CON (1), L-MET (2; 0.097%), D-MET (3; 0.097%), HMBi (4; 0.125%), HMB (5; 0.098%), 2×HMBi (6; 0.250%), ½HMBi + ½DL-MET (7; 0.063% + 0.049%), and HMB + ISO (8; 0.098% + 0.039%). We investigated the effects of the TRTs with batch culture (BC) fermentation. Protozoa were washed out of fermenters in the CC study but traditional BC inocula contain protozoa. Therefore, rumen fluid was collected from 2 Holstein cows and CC was used to prepare faunated (F) and defaunated (D) inocula for use in BC. In addition to TRTs, BC tubes were dosed with ^{15}N enriched $(NH_4)_2SO_4$ to compare incorporation of $^{15}NH_3$ into MICROBIAL PROTEIN. Each TRT contained 6 replicates (D and F inocula each dosed to 3 replicates) with measurements taken at 0, 2, 8, and 24 h. Measurements included TCA soluble N (SN),

$^{15}NH_3$, NH_3, peptides (PEP; SN–NH_3), ^{15}N enriched non-ammonia-N (NAN), and total NAN.

SN, NH_3, and PEP data were analyzed using the mixed procedure of SAS to determine main effects and interactions (other data in progress). The main effect of time was significant for SN, NH_3, and PEP; inocula for SN (D=23.38, F=25.05 mg/dL) and NH_3 (D=10.50, F=12.28 mg/dL); no other main effects were found. Interactions were found (data not shown) for inocula×TRT×time for NH_3 (2h) and PEP (8, 24h), inocula×time for SN (2, 8h) and NH_3 (2, 8h), and TRT×time for PEP (8, 24h). These data indicate possible differences in MET utilization but data completion will allow for more specific conclusions. Application of this data may improve feed efficiency of rumen microbes and dairy cows, which could reduce feed costs and N pollution.

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Thank you Dr. Firkins, interns, and graduate students of the dairy nutrition lab for always offering your time and instruction. You all showed me the purpose and power of research.

My friends and family who have always loved and guided me, this is just one of the many accomplishments I have achieved because of your help. Mom, Dad, Casey, Jackson, Katlyn, and Christine.

List of Abbreviations

AA	amino acid
BC	batch culture
CP	crude protein
CC	continuous culture
d	day
D	defaunated
DM	dry matter
DMI	dry matter intake
DNA	deoxyribonucleic acid
EMPS	efficiency of microbial protein synthesis
F	faunated
h	hour
HMB	2-hydroxy-4(methylthio) butanoic acid
HMBi	2-hydroxy-4(methylthio) butanoic acid isopropyl ester
ISO	isopropanol
MCP	microbial crude protein
MET	methionine
N	nitrogen
NAN	non-ammonia nitrogen
PEP	peptides
SN	soluble nitrogen

TRT	treatment
VFA	volatile fatty acid

Introduction

A better understanding of rumen microbiology can lead to more effective feeding of dairy cows by reducing the cost of feed and the amount of nitrogen excreted in the waste. Feeding dairy cows a balanced AA profile is essential for maximal milk production. MET is one of the two limiting AA for dairy cows and is therefore very important to provide in adequate amounts to avoid limiting milk production. The dairy cow absorbs AA in her hindgut to be utilized for her energy demands, but nutrients must first escape the rumen to be absorbed in the hindgut.

Rumen microbes degrade AA to utilize in their own cellular functions and MPS. Understanding how rumen microbes use dietary MET to efficiently capture N and reduce N in the waste will provide solutions to the questions addressing the most efficient form of fed MET. Several rumen-protected forms of dietary MET have been studied to observe how these products are able to escape rumen microbial degradation and their incorporation in MPS.

Environmental Drive

A growing concern of modern animal agriculture is the impact of agricultural practices on the environment. For dairy cows, this impact is the carbon footprint and N excretion in manure. Many studies have researched alternative solutions to reducing the impact over time. In the dairy industry, many of the solutions come from a nutritional approach with the combined goal of reducing resources into the cow in order to mitigate waste and pollution.

Increased understanding of nutrition and advancements in technology over the past fifty years has allowed for the same amount of milk production with fewer animals. A study by Capper et al. (2009) showed the evolution of the dairy industry between 1944 and 2007. Comparing the statistics to produce 1 billion kg of milk in 1994 to 2007, the dairy industry has made significant reductions in feed, animal numbers, waste outputs, and the overall carbon footprint.

The technologies and management practices necessary for this reduction in resources and substantially increased efficiency came in part from a greater understanding of the nutritional needs of dairy cows. The composition of the first National Research Council (NRC) publication in 1945 on the nutritional needs of dairy cows allowed more accurate diet formulation. There has also historically been a decreased amount of feed intake per unit of milk resulting from an increased understanding of the ratio of nutrients in feed and overall higher nutritional values of crops *(Archibald et al., 1946; NRC, 2001)*. The amount of cropland needed to produce 1 billion kg of milk has declined as a result of advancements in milk and feed production in 2007 to 10% of the land required for 1 billion kg of milk in 1944 (Capper et al., 2009).

This reduction of land, feed, and animal number to produce the same amount of milk over time demonstrates the impact of nutritional research in a practical application. This success of improved crop and milk production is notable through the dairy industry's greenhouse gas emissions and environmental impacts. The green house gas emissions from dairy production account for <1% of the total US greenhouse gas emissions (Capper et al., 2009). The comparison of greenhouse gas emission and

environmental impact between 1944 and 2007 must be made using an outcome basis. The carbon footprint per kg of milk in 2007 is 37% of the carbon footprint of 1944.

Another approach in the reduction of the carbon footprint and methane emissions is to study the effects of diet on the recycling of NH_3-N (Firkins et al., 2007). Understanding the role of microbes in feed digestion and how different feeds change the recycling capacity of NH_3-N will lower the amount of urinary N (Karnati et al., 2009a; Karnati et al., 2009b). The interaction between protozoa and bacteria as a result of diet manipulation is important to understand to avoid negative consequences in production and animal health (Eschenlauer et al., 2002; Firkins and Yu, 2006; Calsamiglia et al., 2007).

Previous Study

An unpublished study previously conducted in the OSU Dairy Nutrition Lab first examined the actions of HMBi in dual flow continuous culture fermenters. The objective of the study was to determine how HMBi supplementation changed EMPS. The results showed decreased N derived from NH_3-N, decreased VFA production, and increased free MET accumulation for the HMBi treatment. Possible explanations for these results are that HMBi was not readily available to rumen microbes and therefore the microbes relied more heavily on de novo synthesis of AA. If HMBi was not being utilized for MPS; it was instead converted into MET in extracellular pools. This explanation would also account for decreased VFA production because microbes had to reserve a greater amount of carbon skeletons for MPS compared to supplementation with DL-MET. The results of this past study led to the objectives examined in the current study.

Objective

This project sought to better understand cellular utilization of MET in dairy cattle to feed more efficiently and reduce N excretion in manure. The treatments were designed to address unanswered questions from the previous continuous culture study to learn how each form of MET changes microbial N incorporation.

L-MET is more readily incorporated into microbial protein than D-MET because of the difference in transport kinetics between the stereoisomers (Kadner, 1977). D-MET must first be oxidized into KMB and then transanimated into L-MET before it can be incorporated into microbial protein. This same process of stereoisomer availability can also be applied to DL-HMB and DL-HMBi. When feeding DL-MET or DL-HMB, only 50% is readily available for microbial utilization because 50% must be converted to L-MET.

HMBi is even less readily incorporated into microbial protein because it must first be hydrolyzed into HMB and isopropanol before it can go through the process of conversion into L-MET. They hydrolysis of HMBi into HMB and isopropanol may have even more effects on cellular incorporation of HMB because alcohols are known to affect membrane fluidity (Grisham and Barnett, 1973; Hui and Barton, 1973).

Material and Methods

This experiment sought to further explore the results of the previous continuous culture study using a new experimental design and additional treatments. Batch culture was chosen and inoculated with two inocula sources: F (faunated, i.e., containing protozoa) and D (defaunated, no protozoa). F inoculum more closely resembles the

rumen microbial population of the cow. D inoculum was used in the previous study and therefore allows further comparisons of results.

Fermenters were used to prepare both inocula for the batch culture. Inoculum was collected from two Holstein cows as the source of both inocula. Rumen fluid was introduced into one fermenter to prepare the F inoculum and a separate fermenter to prepare the D inoculum. Passage and buffering rates of each fermenter were adjusted independently of one another to retain or efflux protozoa.

Inocula were collected from each fermenter and added to every in vitro tube. Each treatment contained either D or F inocula, a treatment, 0.5 g feed, and $(^{15}NH_4)_2S0_4$ (Nitrogen label). The feed was composed of 0.25 g alfalfa and 0.25 g concentrate. $(^{15}NH_4)_2S0_4$ was added to enrich NH3-N and NAN. Measurements were collected at four time points: 0, 2, 8, and 24 h. There were six replicates, three inoculated with D inocula and three with F inocula, per time point for every treatment.

Eight treatments were developed for this experiment from the percent of substrate DM: CON (1), L-MET (2; 0.097%), D-MET (3; 0.097%), HMBi (4; 0.125%), HMB (5; 0.098%), 2×HMBi (6; 0.250%), ½HMBi + ½DL-MET (7; 0.063% + 0.049%), and HMB + ISO (8; 0.098% + 0.039%). Treatments were modeled after the previous continuous culture study and expanded to study the effects of isopropanol and varying concentrations of HMBi on microbial protein synthesis.

Treatments			
TRT #	TRT Description	Inocola	Conc.
1	CON	F	
		D	
2	L- MET	F	0.097%
		D	0.097%
3	D-MET	F	0.097%
		D	0.097%
4	HMBi	F	0.125%
		D	0.125%
5	HMB	F	0.098%
		D	0.098%
6	2 × HMBi	F	0.250%
		D	0.250%
7	1/2 HMBi + 1/2 dl-MET	F	0.063% + 0.049%
		D	0.063% + 0.049%
8	HMB + Isopropanol	F	0.098% + 0.039%
		D	0.098% + 0.039%

In preparation for each trial, all feed was weighed out and placed in each tube. A total of 198 tubes was used for each trial (with a blank every 12 to 18 tubes). The day of inoculation, all treatment solutions were prepared and dosed to the correct tubes along with ^{13}C-[1C]-methionine and $(^{15}NH_4)_2SO_4$. Labeled MET was included to be .0.1% of total MET in the free pool.

Liquid from each fermenter was collected and poured with a cold media solution in a 1:2 dilution through two layers of cheesecloth into two separate containers (one for D inocula and one for F inocula). Carbon dioxide was bubbled into the inocula as it was brought to 39 degrees Celsius in a hot water bath. Each inoculum was continuously hand mixed as 30 ml was added to the appropriate in vitro tube. Each tube was capped with a one-way rubber stopper as soon as incoula was added to maintain an anaerobic environment. For the 0-h time point, 1 mL of 6 N HCl was added to halt fermentation by lowering pH to below 2. Tubes designated for 2- and 8-h measurements were put in a shaking water bath. The 24-h tubes were placed in an incubator until the 8-h tubes had been processed and were then placed in the shaking hot water bath. For the first two hours, tubes were shaken every 20 minutes to redistribute feed particles and were then shaken every 8 hours. Fermentation was halted at each time point by adding 1 mL of 6 N HCl. Tubes were placed in the refrigerator to await further analysis.

Measurements of the supernatant and the pellet provided insight into MPS and N utilization of each treatment. One tube per replicate/treatment/time point was centrifuged at 20,000 x g, and the precipitate was retained. The pH of the precipitate was increased to at least 9 using NaOH and then dried at 55 degrees Celsius for at least four days. After the precipitates were completely dried, the samples were weighed

and ground using mortar and pestle. A 5-mg aliquot was weighed out and sealed in an aluminum capsule for NA^{15}N and total NAN analyses completed at Pennsylvania State University.

TCA soluble N and ammonia determination were analyzed from the supernatant from the tubes. The supernatant was first filtered through two layers of Whatman #1 filter paper. 0.1 mL was removed for ammonia determination and 20 mL was combined with 5 mL of 50% TCA for ^{15}N diffusion (Hristov et al., 2001) and TCA soluble N (Griswold et al., 2003) analyses completed at Pennsylvania State University.

Results and Discussion

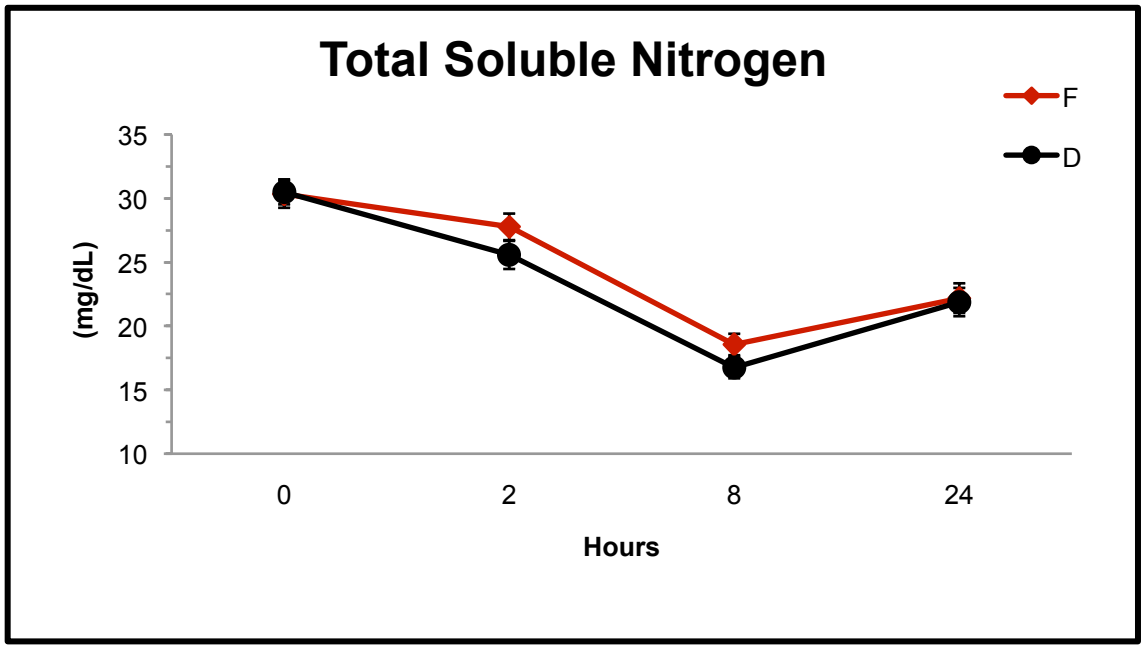

Figure 1. Inocula Effect on Total Soluble N Concentration

Mean total Soluble N (TCA) concentration were different between incoula at 2h ($P < 0.04$) and 8 h ($P < 0.003$)and were not different at 0 and 24 h.

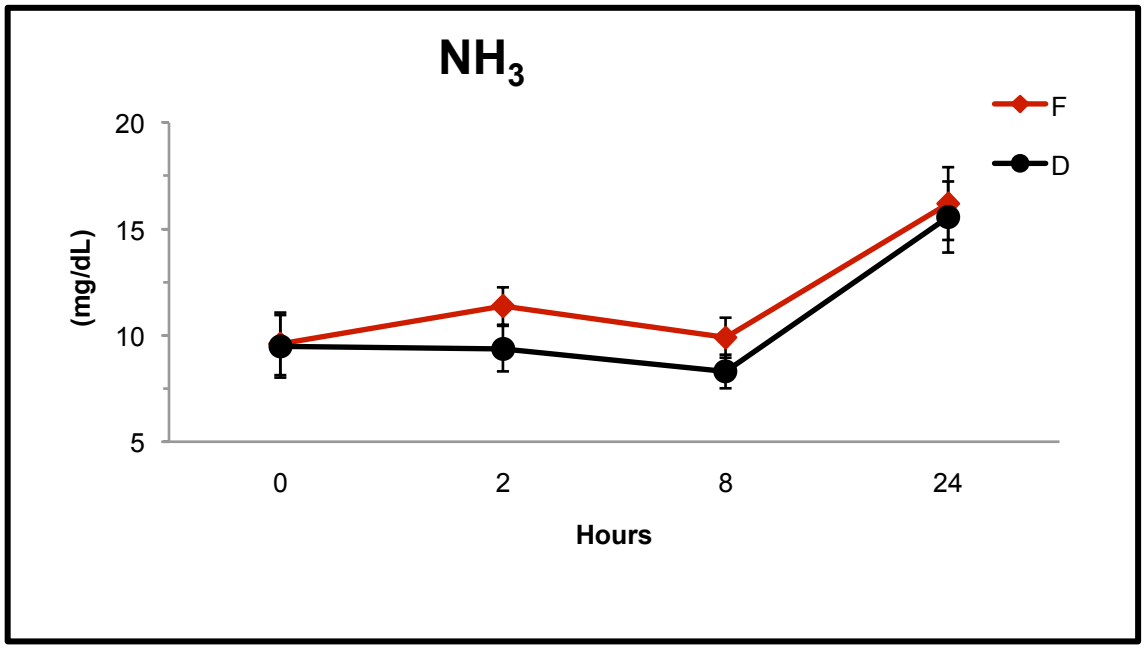

Figure 2. Inocula Effect on NH_3 Concentration

Mean NH_3 concentration were constant t 0 h and then differed between iinocula at 2 and 8 h and were again constant at 24h.

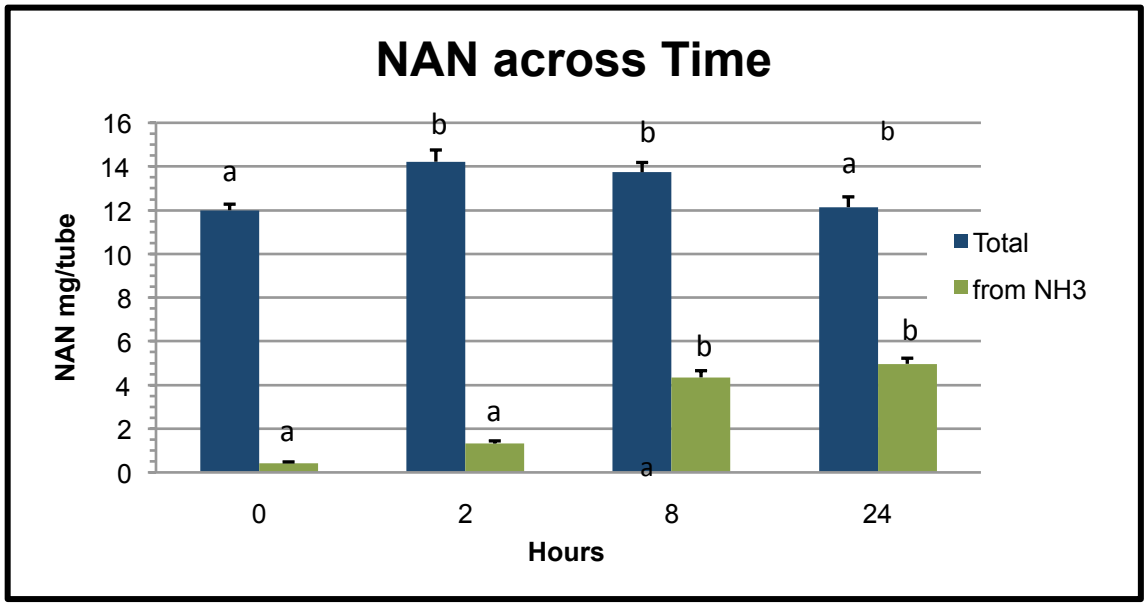

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Figure 3. Effect of Time on Total NAN and NAN Derived From NH_3

Total non-ammonia N (NAN) increased and then decreased back to baseline by 24 h. The amount of NAN produced from NH_3-N increased from 2 to 8 h but not significantly from 8 to 24 h (main effect; $P < 0.001$).

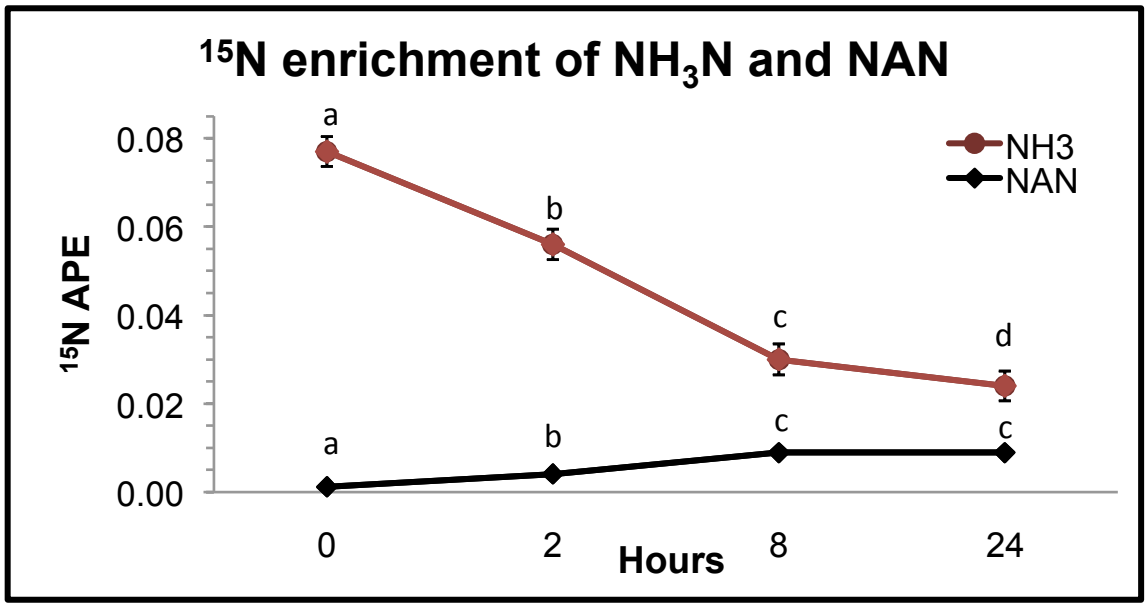

Figure 4. Effect of Time on ^{15}N Enrichment of NH_3N and NAN

Mean ^{15}N enrichment of NH_3 decreased from 0 – 24h as mean ^{15}N enrichment of NH_3 increased from 0-8h and stayed constant from 8-24h (main effect; $P < 0.001$).

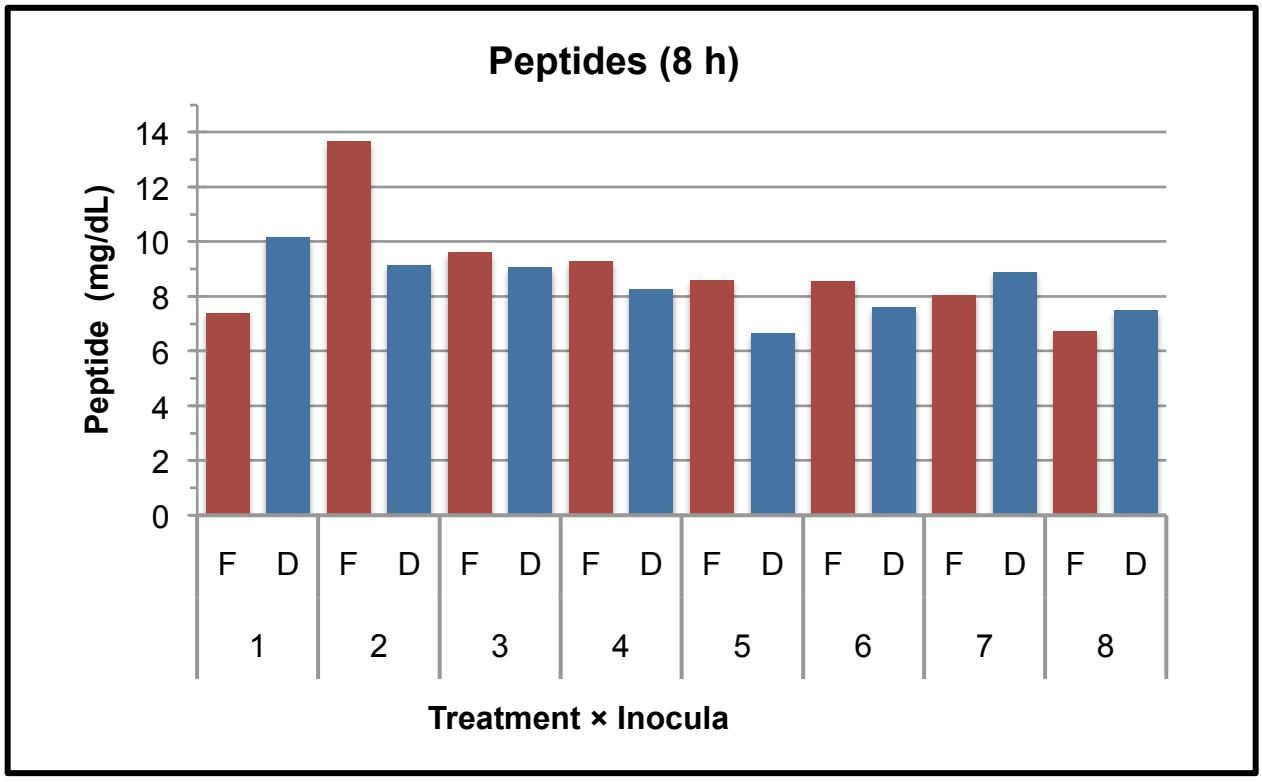

Figure 5. Peptide Concentration: Treatment × Inocula (8h)

L-MET (F) peptide concentration of 13.7 mg/dL was greater than all other treatments. CON (F) peptide concentration of 7.4 also differed from tat of HMB (D) and HMB+ISO (F), which were 6.7 and 6.74 mg/dL, respectively. Peptide concentration for all other treatments averaged 8.7 mg/dL. Peptide concentration for treatment 2 (F) were greater than all other treatments. Peptide concentration for treatment 1 (D) were greater than treatments 5 & 7 (D).

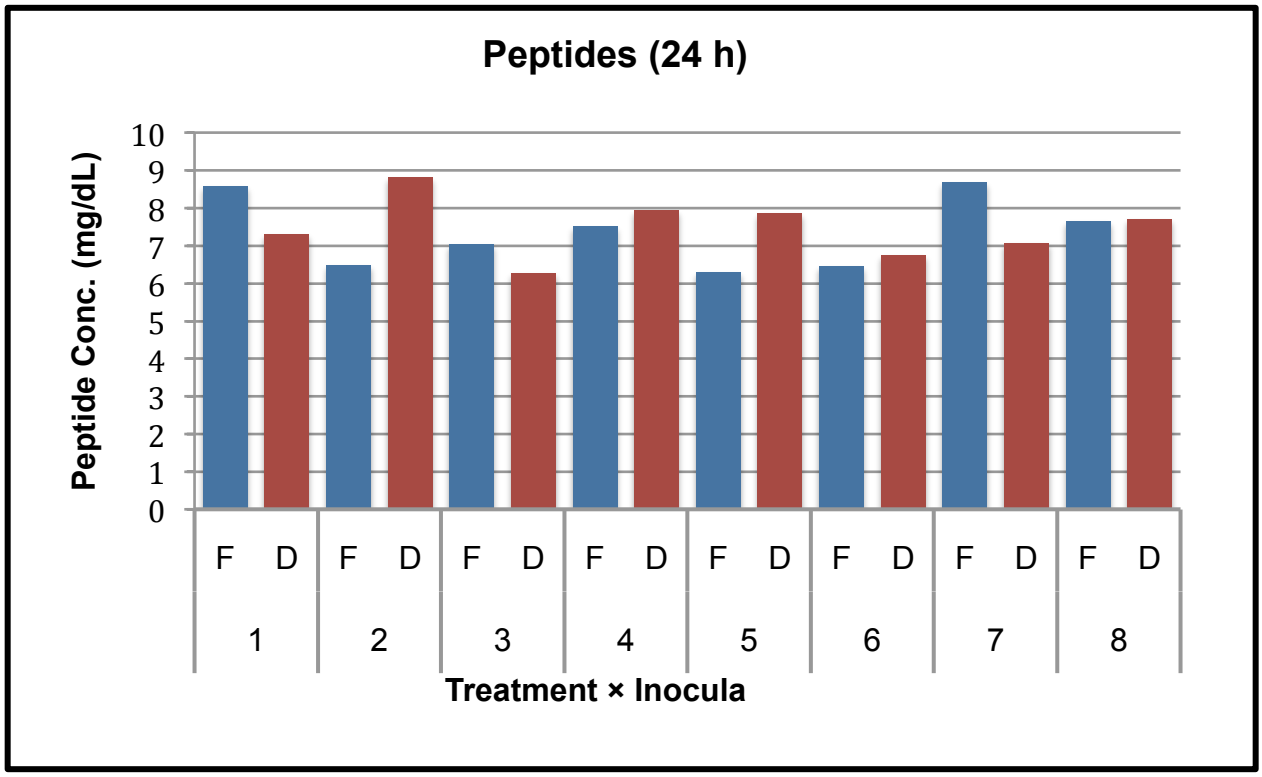

Figure 6. Peptide Concentration for Faunated and Defaunated Treatments (24h)

Peptides for TRT 3 (D) & 5 (F) are lower than TRT 2, 4, 5, 8 (D) & 1, 7, & 8 (F).

Peptides for TRT 2 (D) & 7 (F) are greater than TRT 1, 3, 6, 7 (D) and 2, 3, 5, & 6 (F)

Conclusion

The results from this study are still incomplete until the analysis of data comparing MET enrichment. The main effect found in the results was time. Comparing incocula, F inocula contain greater concentrations of N over time than D inocula, as shown in TCA soluble-N and NH_3 concentrations.

Total non-ammonia nitrogen (NAN) was greatest at 2 h because microbial protein was being produced rapidly and feed protein had not been extensively degraded. With increasing time, feed protein was degrading and fluxing through the NH_3-N pool and being synthesized into microbial NAN. Comparing the NH_3-N transfer into NAN peak at 8 h and peptide concentration decreased by 24 h, bacteria were growing and producing new protein in initial phases but were using more recycled N from microbial cells from the inoculum by 24 h.

Further treatment comparisons cannot be made with incomplete data. It can be predicted from this data that treatments show potential to alter rate of ^{15}N incorporation into protein at incubation length of 8 and 24 hours. Continued analysis and understanding of the form of MET supplementation on microbial protein synthesis is dependent on the analysis of cellular MET incorporation.

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